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Characterization of a Resistance-Nodulation-Cell Division Transporter System Associated with the *syr-syp* Genomic Island of *Pseudomonas syringae* pv. syringae

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A tripartite resistance-nodulation-cell division (RND) transporter system, called the PseABC efflux system, was identified at the left border of the *syr-syp* genomic island of *Pseudomonas syringae* pv. syringae strain B301D. The PseABC efflux system was located within a 5.7-kb operon that encodes an outer membrane protein (PseA), a periplasmic membrane fusion protein (PseB), and an RND-type cytoplasmic membrane protein (PseC). The PseABC efflux system exhibited amino acid homology to a putative RND efflux system of *Ralstonia solanacearum*, with identities of 48% for PseA, 51% for PseB, and 61% for PseC. A nonpolar mutation within the *pseC* gene was generated by *nptII* insertional mutagenesis. The resultant mutant strain showed a larger reduction in syringopeptin secretion (67%) than in syringomycin secretion (41%) compared to parental strain B301D ($P < 0.05$). A β-glucuronidase assay with a *pseA*::*uidA* reporter construct indicated that the GacS/GacA two-component system controls expression of the *pseA* gene. Quantitative real-time reverse transcription-PCR was used to determine transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes in strain B301D-HK4 (a *pseC* mutant). The expression of the *sypA* gene by mutant strain B301D-HK4 corresponded to approximately 13% of that by parental strain B301D, whereas the *syrB1* gene expression by mutant strain B301D-HK4 was nearly 61% ($P < 0.05$). In addition, the virulence of mutant strain B301D-HK4 for immature cherry fruits was reduced by about 58% compared to parental strain B301D ($P < 0.05$). Although the resistance of mutant strain B301D-HK4 to any antibiotic used in this study was not reduced compared to parental strain B301D, a drug-supersensitive *acrB* mutant of *Escherichia coli* showed two- to fourfold-increased resistance to acriflavine, erythromycin, and tetracycline upon heterologous expression of the *pseA*, *pseB*, and *pseC* genes (*pseABC* efflux genes). The PseABC efflux system is the first RND transporter system described for *P. syringae*, and it has an important role in secretion of syringomycin and syringopeptin.

Pseudomonas syringae pv. syringae is a common plant bacterial pathogen in nature that causes necrosis in a wide spectrum of monocot and dicot plants (9). A distinctive characteristic of *P. syringae* pv. syringae is its secretion of two different classes of lipopeptide phytotoxins, called syringomycins and syringopeptins (7). The major form of syringomycin, SRE, is a cyclic nonapeptide attached to a 3-hydroxydodecanoic acid tail. In contrast, the major form of syringopeptin, $SP_{22}B$, contains a cyclic peptide with 22 amino acids attached to a 3-hydroxydodecanoic acid tail. The mode of action of the phytotoxins is to cause cellular lysis by the formation of transmembrane pores in the plasma membranes of host cells that lead to disruption of the membrane electrical potential (26). The phytotoxins are encoded by the *syr-syp* genomic island spanning approximately a 155-kb DNA region, which corresponds to over 2% of the genome of *P. syringae* pv. syringae strain B301D (22, 60). The *syr-syp* genomic island consists of genes required for phytotoxin biosynthesis, secretion, and regulation (7, 35, 52, 60).

Type I secretion systems are characterized by a one-step transport process and are ubiquitous among gram-negative bacteria, including *Pseudomonas* and *Xanthomonas* (53). For example, *P. syringae* pv. tomato DC3000, whose genome sequence was released recently (10), possesses 15 ATP-binding cassette (ABC) transport systems and nine resistance-nodulation-cell division (RND)-type efflux systems (10). These transport systems are predicted to be involved in sugar transport (ABC) and the export of drugs or cations (RND). In addition, in *P. syringae* the type I secretion system is known to be essential for biosynthesis and transport of secondary metabolites in this gram-negative bacterium. *Pseudomonas aeruginosa* PAO produces pyoverdine, a siderophore whose secretion requires a protein homologous to an ABC transporter called PvdE (37). The *Xanthomonas oryzae* pigment, xanthomonadin, is localized to the outer membrane by a putative RND-type transporter (20). Thus, type I secretion systems are required for the export of a variety of metabolic products.

Certain families of the RND-type transporter superfamily form a functional three-component efflux system with a membrane fusion protein and an outer membrane protein (51, 53, 64). The resultant efflux system is proposed to utilize a dual entrance to pump out hydrophobic and hydrophilic substrates from the cytoplasmic membrane or the periplasm to the external environment (40). The most intensively studied RND-type efflux system is the AcrAB-TolC efflux system found in *Escherichia coli* K-12 (46) and *Salmonella enterica* serovar Typhimurium SH5014 (44). The AcrAB-TolC efflux system plays an important role in bacterial resistance by exporting various compounds, such as acriflavine, antibiotics, and lipophilic molecules. AcrA is a periplasmic protein anchored to the inner

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membrane. AcrB is an RND transporter composed of 12 transmembrane α-helices and two large hydrophilic loops. TolC is an outer membrane protein that forms a channel that allows antibiotics to diffuse across the outer membrane. However, despite the prevalence of RND-type efflux systems in gram-negative bacteria (70), little is known about the RND-type efflux system involved in the secretion of lipopeptide toxins produced by gram-negative bacteria.

The secretion of lipopeptide toxins is essential for toxigenesis by *P. syringae* pv. syringae (52). The *syrD* gene is located between the *syr* and *syp* genomic islands. Based on an analysis of the sequence, the SyrD protein is homologous to a cytoplasmic membrane protein belonging to the ABC transporter family (57). In strain BR105 (a *syrD* mutant) secretion of syringomycin and syringopeptin is significantly reduced (52). Mutant strain BR105 showed a greater reduction (70%) in virulence for immature sweet cherry fruits than syringomycin synthetase mutant BR132 (a *syrB1* mutant; 26% reduction) and syringopeptin synthetase mutant B301D-208 (a *sypA* mutant; 59% reduction) compared to parental strain B301D (58). The reduction in virulence was attributed to a decrease in secretion of both syringomycin and syringopeptin. However, mutation of the *syrD* gene failed to cause a complete loss of secretion of lipopeptide phytotoxins (21), which led to speculation that the SyrD protein is not the sole transport system responsible for secretion of syringomycin and syringopeptin.

In this study, a tripartite RND-type efflux system, called the *P. syringae* syringomycin and syringopeptin efflux system (PseABC efflux system), was identified at the left border of the *syr-syp* genomic island of *P. syringae* pv. syringae strain B301D by sequencing cosmid JS115. The PseABC efflux system was hypothesized to have a role in secretion of syringomycin and syringopeptin. The objective of this study was to elucidate the function of the putative RND-type efflux system and its contribution to virulence in *P. syringae* pv. syringae strain B301D. Studies of the PseABC efflux system demonstrated that disruption of the *pseC* gene caused a large reduction in syringopeptin secretion, a limited reduction in syringomycin secretion, and a substantial reduction in the virulence of *P. syringae* pv. syringae strain B301D.

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used in this study are listed in Table 1. *E. coli* strain DH10B (Gibco-BRL), which was used for DNA manipulations, was cultured at 37°C in Luria-Bertani broth or on Luria-Bertani agar (55). *P. syringae* pv. syringae strains were cultured routinely in nutrient broth yeast extract liquid or agar medium (65). Potato dextrose agar (PDA) supplemented with 0.4% Casamino Acids and 1.5% glucose (23) was used in bioassays for production or secretion of syringomycin and syringopeptin. When required, antibiotics (Sigma) were added to media at the following final concentrations: 100 μg/ml of ampicillin (*E. coli*), 50 μg/ml of kanamycin (*E. coli* and *Pseudomonas*), 10 μg/ml of gentamicin (*E. coli*), 200 μg/ml of chloramphenicol (*E. coli* and *Pseudomonas*), and 6.25 to 25 μg/ml of tetracycline (*E. coli* and *Pseudomonas*).

DNA manipulations and sequence analysis. Routine procedures (56) were used for plasmid isolation from *E. coli*, restriction endonuclease digestion, and subcloning. An 8.4-kb KpnI fragment from pJS115 containing the *pseABC* efflux genes and a 4.7-kb HindIII fragment from pJS091 containing the *pseA* gene were sequenced (59). Sequence data were analyzed using the Wisconsin Sequence Analysis programs of the Genetic Computer Group package, version 10.0 (13), and Lasergene expert sequence analysis software (version 5.0; DNASTAR). The Genetic Computer Group programs FINDPATTERNS and TERMINATOR were used to identify Shine-Dalgarno sequences and to predict rho-independent transcriptional terminators. Sequence randomization and calculation of Z scores were performed using the GAP program, which evaluates the significance of protein sequence similarity as described previously (59). Protein sequence similarity was considered to be significant and to indicate homology when the Z score was greater than 6. Database searches for genes and proteins were performed using the BLAST servers of the National Center for Biotechnology Information (http://www.ncbi.nih.gov) and the Transporter Protein Analysis Database server (http://66.93.129.133/transporter/wb/index2.html) (5). A motif search was performed using the Pfam server (http://motif.ad.jp/motif-bn/Srch_Motif_Lib) (6). Hydropathy analysis was performed to predict transmembrane segments (TMSs) using Protean (Lasergene) and the hydropathy analysis server (http://megaman.ucsd.edu/progs/hydro.php) (32). Multiple alignment of nucleotide or protein sequences was performed using the MegAlign program (Lasergene) and the MultiAlign server (http://prodes.toulouse.inra.fr/multalin/multalin.html) (12).

Mutagenesis. The *pseA*, *pseB*, and *pseC* genes were disrupted by insertion of the *nptII* gene (3). A 1.2-kb *nptII* cassette from pBSL15 was inserted into the PmlI site of the *pseA* gene in pJS091, into the EcoRV site of the *pseB* gene in pHK01, and into the AgeI site of the *pseC* gene in pHK01. The resultant *pseA*::*nptII*, *pseB*::*nptII*, and *pseC*::*nptII* constructs were subcloned into the EcoRV site of pBR325, yielding plasmids pHK22, pHK32, and pHK42, respectively. To allow marker exchange mutagenesis to occur, *P. syringae* pv. syringae strain B301D was transformed with pHK22, pHK32, and pHK42 by electroporation using a Gene Pulser II (Bio-Rad Laboratories) as described previously (58). Transformants were selected on nutrient broth yeast extract agar supplemented with kanamycin. Double crossover mutations were confirmed by Southern analysis and by PCR. The confirmed *pseA*, *pseB*, and *pseC* mutants (*pseABC* mutants) were designated B301D-HK2, B301D-HK3, and B301D-HK4, respectively. A *syrB1* and *pseC* double mutant (BR132-HK4) was generated by marker exchange of *pseC*::*nptII* into the genome of *syrB1* mutant strain BR132 (69). Plasmid pHK115, which carried the *pseABC* efflux genes, was introduced into the *pseABC* mutants in order to test for complementation of the *pseA*, *pseB*, and *pseC* mutations.

To generate a *gacA* mutant, the region flanking 1.0 kb to the 5′ terminus of the *gacA* gene and 0.6 kb to the 3′ terminus of the *gacA* gene was amplified from genomic DNA of parental strain B301D by PCR using Vent polymerase (New England Biolabs). The amplified *gacA* gene was cloned into the pGEM-T Easy vector (Promega) to create plasmid pHK51. Plasmid pHK51 was digested with EcoRV to insert the *nptII* gene cassette, which resulted in plasmid pHK52. Plasmid pHK52 was digested with NotI to release a 3.5-kb fragment. The resultant 3.5-kb NotI fragment was polished with T4 DNA polymerase and inserted into the EcoRV site of pBR325 to construct plasmid pHK53. Plasmid pHK53 was introduced into the genome of parental strain B301D to generate the *gacA* mutant by marker exchange mutagenesis.

Site-directed mutagenesis (68) was performed to replace Lys^{906} with Asp in the *pseC* gene of pHK115 (24). The sequence of mutagenic primer K906D was as follows: 5′-GGGGATCGTGACCGACAACTCGTACCTGCTG-3′. The primer pair used to introduce the point mutation was antiparallel and overlapping. PCR products resulting from the mutant strand synthesis reaction were treated with DpnI to digest the *dam*-methylated template plasmid. The resultant unmethylated PCR products were directly transformed into *E. coli*. The correct point mutation was verified by DNA sequence analysis (31). The resultant mutant construct of pHK115 (K906D) was designated pHK116.

Quantitative real-time RT-PCR. Quantitative real-time reverse transcription-PCR (RT-PCR) was used to determine the effect of a *pseC* mutation on expression of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes (18). Bacterial RNAs were extracted using an RNeasy Mini kit (QIAGEN) from *P. syringae* pv. syringae B301D (parental strain) and B301D-HK4 (*pseC* mutant strain) grown on SRM_{AF} medium at 25°C for 72 h (39). Purified RNA was prepared according to the manufacturer's instructions, which required DNase digestion using an RNase-free DNase set (QIAGEN). Oligonucleotide primers were designed by using the PrimerSelect software (version 5.0; DNASTAR).

Reaction components were prepared according to the manufacturer's instructions, except that each reaction was set up in 25 μl with 100 ng of template RNA and 1.25 pmol of each primer. The RT reaction was performed for 30 min at 94°C with 30 s of primer annealing at 54°C, followed by 45 cycles of 15 s of denaturation at 94°C, 30 s of primer annealing at 54°C, and 30 s of polymerization at 60°C. Primers were evaluated by following the manufacturer's instructions (QIAGEN). The fold induction of mRNA was determined from the threshold values that were normalized for 16S rRNA gene expression (endogenous control) and then normalized to the threshold value obtained for parental strain B301D (4).

Before expression profiles of the *syrB1* and *sypA* genes were determined, the relative amplification efficiencies of the *syrB1*, *sypA*, and 16S primer pairs were

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant characteristics[a]	Source or reference
E. coli strains		
DH10B	F$^-$ *mcrA* Δ*lacX74* (ϕ80d*lacZ*ΔM15) Δ(*mrr-hsdRMS-mcrB*) *deoR recA1 endA1 araD139* Δ(*ara leu*)*7697 galU galK*λ$^-$ *rpsL nupG*	Invitrogen
AG100	*argE3 thi-1 rpsL xyl mtl galK supE441* Δ(*gal-uvrB*) λ$^-$	46
AG100A	Kan^r, same as AG100 but Δ*acrAB*::*kan*	46
P. syringae pv. syringae strains		
B301D	Wild type from pear	11
BR132	*syrB1*::Tn*3*HoHo1 derivative of B301D-R; Pip^r Rif^r	38
B301D-208	*sypA*::Tn5 derivative of B301D; Km^r	This study
B301D-HK2	*pseA*::*nptII* derivative of B301D; Km^r	This study
B301D-HK3	*pseB*::*nptII* derivative of B301D; Km^r	This study
B301D-HK4	*pseC*::*nptII* derivative of B301D; Km^r	This study
BR132-HK4	*pseC*::*nptII* derivative of BR132; Pip^r Rif^r Km^r	This study
B301D-HK5	*gacA*::*nptII* derivative of B301D; Km^r	This study
B301D-SL7	*salA*::*nptII* derivative of B301D; Km^r	35
P. aeruginosa PAO1	Wild type	29
Plasmids		
pBSK(+)	Cloning vector; Ap^r	Strategene
pBR325	Cloning vector; Cm^r Tc^r Ap^r	50
pSL02	pBI101 carrying the 0.85-kb *aacCI* gene from pUCGM inserted into the EcoRI site downstream of the *uidA* gene; Km^r Gm^r	35
pGEMT-Easy	Cloning vector; Ap^r	Promega
pUCP26	Cloning vector; Tc^r	47
pBSL15	Kanamycin resistance gene cassette; Km^r	3
pJS091	pBSK carrying a 4.7-kb HindIII fragment from strain B301D; Ap^r	62
pHK091	pUCP26 carrying the 4.7-kb HindIII fragment from pJS091, Tc^r	This study
pHK092	pUCP26 carrying the 4.7-kb HindIII fragment from pHK091 with the 3.2-kb *uidA-aacCI* fragment from pSL02 inserted into the PmlI site in-frame with *pseA* in forward orientation; Tc^r Gm^r	This study
pJS115	pBSK carrying an 8.4-kb KpnI fragment from strain B301D; Ap^r	60
pHK115	pUCP26 carrying the 8.4-kb KpnI fragment from pJS115	This study
pHK116	pHK115 having site-directed mutagenesis in the *pseC* gene	This study
pHK01	pGEM T-Easy carrying the 5.9 kb PvuII-ScaI fragment from pJS115 at the EcoRV site	This study
pHK21	pJS091 carrying the 1.2-kb *nptII* gene from pBSL15 inserted into the PmlI site of *pseA*	This study
pHK22	pBR325 carrying the 3.6-kb HindIII-StuI fragment from pHK21 at the EcoRV site	This study
pHK31	pHK01 carrying the 1.2-kb *nptII* gene of pBSL15 inserted into the EcoRV site of *pseB*	This study
pHK32	pBR325 carrying the 5.0-kb PmlI fragment from pHK31 at the EcoRV site	This study
pHK41	pHK01 carrying the 1.2-kb *nptII* gene of pBSL15 inserted into the AgeI site of *pseC*	This study
pHK42	pBR325 carrying the 6.4-kb ApaI-SpeI fragment of pHK31 at the EcoRV site	This study
pHK51	pGEM T-Easy carrying the 2.4-kb PCR product spanning *gacA* at the EcoRV site	This study
pHK52	pHK51 carrying the 1.2-kb *nptII* gene inserted into the EcoRV site of *gacA*	This study
pHK53	pBR325 carrying a 3.5-kb NotI fragment of pHK52 at the EcoRV site	This study

[a] Pip^r, Rif^r, Cm^r, Tc^r, Ap^r, Km^r, and Gm^r, resistance to piperacillin, rifampin, chloramphenicol, tetracycline, ampicillin, kanamycin, and gentamicin, respectively.

assessed as described in the manufacturer's instructions (33). The differences in amplification efficiency of the primer pairs were less than 0.1, which indicated that the amplification efficiencies were approximately equal. Quantitative real-time RT-PCR was accomplished by using a QuantiTect SYBR Green RT-PCR kit (QIAGEN) and a Smart Cycler (Cepheid). The primers used for quantitative real-time RT-PCR are listed in Table 2. All quantitative real-time PCRs were repeated three times with two plates per replicate.

Screening for syringomycin and syringopeptin secretion by the *pseC* mutant. Strains B301D-HK2 (a *pseA* mutant), B301D-HK3 (a *pseB* mutant), and B301D-HK4 (a *pseC* mutant) were screened for secretion of syringomycin and syringopeptin using standard bioassays as previously reported (58), except that the strains were cultured on PDA plates. To assay syringomycin production, the plates were incubated for 72 h at 25°C, the indicator fungus *Geotrichum candidum* F-260 was oversprayed, and the plates were incubated at 25°C for another 24 h. To assay syringopeptin production, the plates were incubated for 48 h at 25°C, the indicator bacterium *Bacillus megaterium* Km was oversprayed, and the plates were incubated at 25°C for another 24 h. Resultant zones of growth inhibition of *G. candidum* and *B. megaterium* were measured. A low concentration of tetracycline (6.25 μg/ml) was added to the PDA in order to maintain pHK115. The PDA plate bioassays were replicated six times.

Virulence assays with immature cherry fruits. Virulence assays for strains B301D-HK2 (a *pseA* mutant) and B301D-HK4 (a *pseC* mutant) were performed with immature cherry fruits as described previously (58). Each wound site on cherry fruits was inoculated with 5×10^3 CFU of a strain of *P. syringae* pv.

TABLE 2. Primer sequences used for quantitative real-time RT-PCR

Gene	Primer[a]	Sequence
syrB1	F-RT-*syrB1*	TTAGCGCCGCGTCAGCCCCTTCAAG
	R-RT-*syrB1*	GCTCAACGTCCGGGCTGCATCGCTCAC
sypA	F-RT-*sypA*	TGCGGGTCGAGGCGTTTTTG
	R-RT-*sypA*	GTTGCCGCGTCCTTGTCTGA
16S rRNA	F-RT-16S	ACACCGCCCGTCACACCA
	R-RT-16S	GTTCCCCTACGGCTACCTT

[a] F, forward; R, reverse.

syringae. The inoculated fruits were incubated for 4 days at 20°C. Virulence was determined by measuring the diameter of the necrotic lesion formed at each inoculation site. For each experiment, 10 cherry fruits were inoculated per treatment, and the experiment was repeated three times. Parental strain B301D and strain BR132 (a *syrB1* mutant) were used as controls.

Construction of GUS fusions and GUS assay. The *uidA* gene encoding β-glucuronidase (GUS) was inserted into the *pseA* gene in frame to determine expression of the *pseA* gene in *P. syringae* pv. syringae strains (45). Digestion of plasmid pSL2 (35) with HindIII and BglI was used to clone the *uidA-aacCI* reporter from pSL2 into JS091 (60). The resultant 3.2-kb HindIII-BglI fragment containing the *uidA-aacCI* reporter was polished with T4 DNA polymerase (New England Biolabs); the polished 3.2-kb HindIII-BglI fragment was inserted in frame into the PmlI site of the *pseA* gene to generate pHK091. Plasmid pHK091 was digested with HindIII and StuI to recover a 5.6-kb HindIII-StuI fragment. The 5.6-kb fragment was polished with T4 DNA polymerase and inserted into the SmaI site of pUCP26 (47) in the reverse orientation to generate pHK092. Sequence analysis of the *pseA*::*uidA* reporter construct verified production of a truncated PseA protein fused with GUS. GUS activity was measured using previously reported methods (61). One unit of activity was defined as cleavage of 1 pmol of *p*-nitrophenyl-β-D-glucuronide per min per bacterium (1). All assays for GUS activity were repeated on four independent occasions.

MIC tests. The susceptibilities to acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin, and novobiocin (Sigma) were tested using a microtiter broth dilution method (34). The susceptibilities of strain B301D-HK4 (a *pseC* mutant) were compared with those of parental strain B301D and *P. aeruginosa* PAO1 (29). In addition to *Pseudomonas* spp., *E. coli* strains AG100A (an *acrB* mutant) and AG100 (*acrB*$^+$ parent) were transformed with pJS115, which carries the *pseA*, *pseB*, and *pseC* genes (60). The resultant transformed *E. coli* strains were tested to determine whether the PseABC efflux system enhanced the resistance of mutant strain AG100A to antibiotics. Parental strain AG100 was used as a control. Briefly, exponential-phase bacterial cells were added to a sterile 96-well microtiter plate containing Mueller-Hinton broth and serial twofold dilutions of antibiotics (14). Modification of the MICs was observed for mutant strain AG100A when the strain was transformed with pBSK (control vector). The resultant AG100A strain carrying the pBSK vector was more susceptible to certain antibiotics (tetracycline and acriflavine) than mutant strain AG100A without the pBSK vector was. The final cell concentration was adjusted to 5×10^4 CFU/ml per well. The *E. coli* strains were incubated at 37°C for 12 h. The *Pseudomonas* strains were cultured at 25°C for 18 h. The MIC was defined as the lowest concentration of antibiotic that inhibited visible growth (62), which was confirmed by measuring the optical density at 600 nm of a cell suspension grown in a 96-well plate. All assays for MIC were repeated on four independent occasions.

Statistical analysis. Means in each test were compared with one another by using a *t* test and the Tukey W procedure (48).

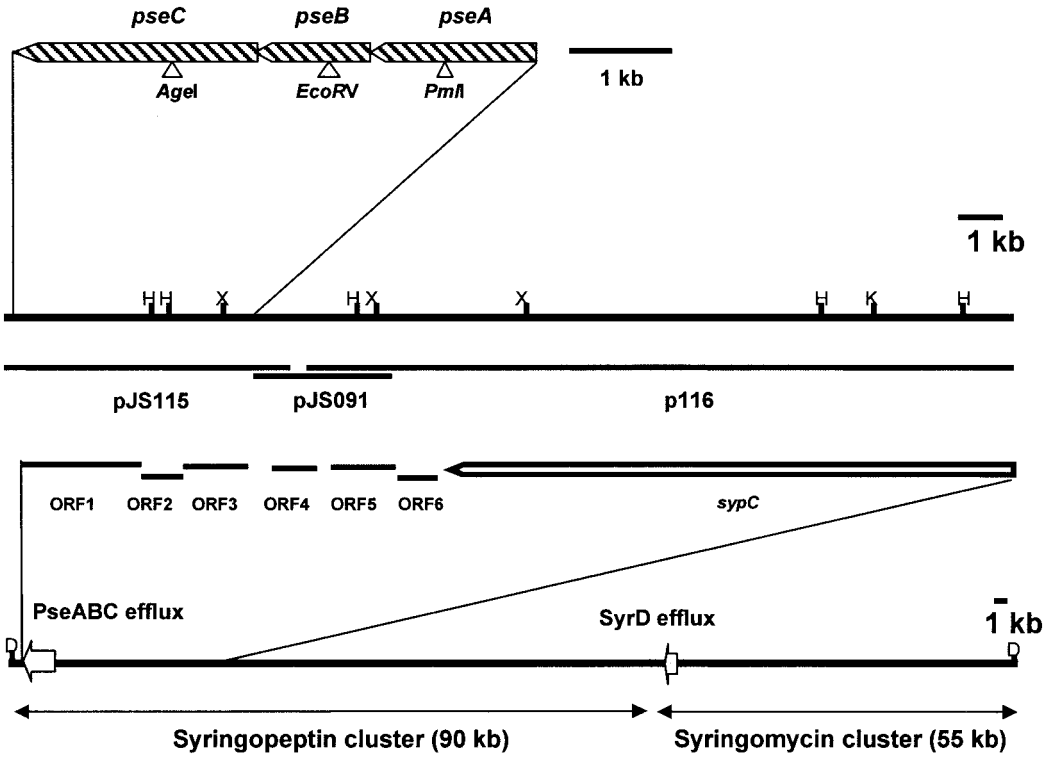

FIG. 1. Diagrammatic representation of the *syr-syp* genomic island on the chromosome of *P. syringae* pv. syringae B301D. The approximately 145-kb DraI fragment consists of the syringopeptin (*syp*) gene cluster (90 kb) and the syringomycin (*syr*) gene cluster (55 kb) (60). The left border of the *syp* gene cluster, a 51-kb region, is shown above the diagram of the DraI fragment. The positions of the *pseA* gene (ORF3, 1.5 kb), the *pseB* gene (ORF2, 1.1 kb), and the *pseC* gene (ORF1, 3.0 kb) are indicated on the map of the 51-kb region. Mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 were generated by disrupting the *pseA* (ORF1), *pseB* (ORF2), and *pseC* (ORF3) genes, respectively, by *nptII* insertional mutagenesis. The triangles indicate the restriction sites into which the *nptII* cassette was inserted in the *pseA*, *pseB*, and *pseC* genes. The open arrows in the *syr-syp* genomic island represent the locations of the PseABC and SyrD efflux systems. The restriction enzyme sites are indicated as follows: D, DraI; H, HindIII; K, KpnI; X, XhoI.

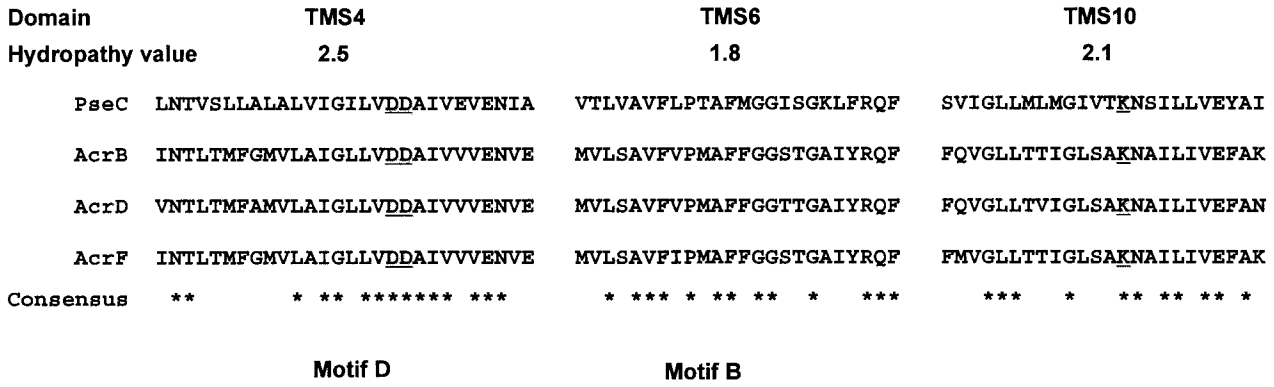

FIG. 2. Amino acid sequence alignment of TMS4, TMS6, and TMS10 of representative RND transporters (51). Motifs D and B that are characteristic of the AcrB, AcrD, and AcrF RND transporters were found in TMS4 and TMS6 of the PseC protein, respectively. The hydropathy values for TMS4, TMS6, and TMS7 of the PseC protein are indicated above the corresponding amino acid residues. Consensus amino acid residues are indicated by asterisks. The amino acid residues that are underlined are the conserved aspartic acid (D) and lysine (K) residues that are essential residues in TMS4 and TMS10 of the MexB (24) and AcrB (40) proteins. These D and K residues were identified in TMS4 and TMS10 of the PseC protein.

RESULTS

Sequence analysis of the *pseA*, *pseB* and *pseC* genes. Sequencing of p116, pJS091, and pJS115 revealed six open reading frames (ORFs) downstream of the *sypC* gene (60) (Fig. 1). ORF1, ORF2, and ORF3 encoded three components of the PseABC efflux system, which was composed of an outer membrane protein, a periplasmic membrane fusion protein, and a cytoplasmic RND transporter (54). ORF4 encoded a probable class III aminotransferase (27, 60). ORF5 and ORF6 encoded an ABC transporter homolog and a periplasmic membrane fusion protein, respectively (60). The stop codon (TGA) of ORF1 overlapped the start codon (ATG) of ORF2 by 4 bp. Similarly, a 4-bp overlap was observed between the stop codon of ORF2 (TGA) and the start codon (ATG) of ORF3.

The protein encoded by ORF3, PseA, was 518 amino acids long and was predicted to encode an outer membrane protein. The PseA protein showed 48.2% identity (Z score, 142) to an outer membrane protein of *Ralstonia solanacearum* GMI1000 (55), 32.6% identity (Z score, 54) to the OprM protein of *P. aeruginosa* PAO1 (63), and 23.9% identity (Z score, 11) to the TolC protein of *E. coli* K-12 (8). A probable Shine-Dalgarno sequence (AGGCGT) was predicted to be 9 bp upstream of the start codon (ATG) of the *pseA* gene. A rho-independent transcriptional terminator was not found downstream of the stop codon (TGA) of the *pseA* gene. A motif search predicted that the PseA protein contained two motifs characteristic of the outer membrane efflux protein family (28). The first motif corresponded to residues 107 to 292 of the PseA protein (E value, 1.9e-14), whereas the second motif was located between residues 317 and 498 of the PseA protein (E value, 2.1e-43). These motifs exhibited heptad repeat patterns that are suggestive of coiled-coil structures (28). Helices of the outer membrane efflux protein family have been shown to contain coiled-coil structures, and they have been proposed to form a transient complex with periplasmic efflux proteins (membrane fusion protein) for the export of substrates (28). Hydropathy analysis (51) predicted that the N terminus of the PseA protein contained two TMSs.

The protein encoded by ORF2, PseB, was 367 amino acids long and was predicted to encode a periplasmic membrane fusion protein. The PseB protein showed 51.2% identity (Z score, 87) to a putative membrane fusion protein of *R. solanacearum* GMI1000 (55), 23.9% identity (Z score, 9) to the MexA protein of *P. aeruginosa* PAO1 (63), and 23.4% identity (Z score, 9) to the AcrA protein of *E. coli* K-12 (8). A probable Shine-Dalgarno sequence (GGGCGG) was identified 9 bp upstream of the start codon (ATG) of the *pseB* gene. No rho-independent transcriptional terminator was identified downstream of the stop codon (TGA) of the *pseB* gene. A motif search predicted that the PseB protein had a hemolysin D (HlyD) family secretion protein signature (19). The HlyD protein is a member of the membrane fusion protein family (28). The signature corresponded to residues 66 to 200 of the PseB protein (E value, 2.9e-05), and it has been suggested that it is associated with a periplasmic efflux protein (membrane fusion protein) to form a bridge between an outer membrane protein and a cytoplasmic efflux protein (28). PseB protein was predicted to contain one TMS at the N terminus by hydropathy analysis (51).

The RND-type transporter encoded by ORF1, PseC, was predicted to be 1,009 amino acids long. The PseC protein showed 61.6% identity (Z score, 563) to a probable transporter transmembrane protein of *R. solanacearum* GMI1000 (55), 28.3% identity (Z score, 134) to the MexB protein of *P. aeruginosa* PAO1 (63), and 27.2% identity (Z score, 9) to the AcrB protein of *E. coli* K-12 (8). A probable Shine-Dalgarno sequence (AGGCCC) was located 13 bp upstream of the start codon (ATG) of the *pseC* gene. The primary structure of a rho-independent transcriptional terminator was observed 108 bp downstream of the stop codon (TGA) of the *pseC* gene (primary structure value, 3.52). A motif search predicted that the PseC protein contained four AcrB/AcrD/AcrF family motifs (motifs A to D) (51). The AcrB/AcrD/AcrF family (cytoplasmic membrane protein) is one of the most well-studied RND-type transporter families (51). These motifs were dispersed within amino acid residues 3 to 996 of the PseC protein (E value, 3e-215). Motif A was located in a loop between TMS1 and TMS2 of the PseC protein, and it is predicted to be

TABLE 3. Production of syringomycin and syringopeptin by *P. syringae* pv. syringae strains B301D and B301D-HK4 (a *pseC* mutant)

Strain	Bioassay on PDA (mm)[a]	
	Syringomycin	Syringopeptin
B301D	11.2 ± 1.2[b]	9.2 ± 1.1
B301D-HK4	6.6 ± 1.2	3.7 ± 0.7
B301D-HK4(pHK115)	9.4 ± 0.9	7.7 ± 1.0

[a] Syringomycin production and syringopeptin production were assessed by measuring the radius of inhibition zones of *G. candidum* and *B. megaterium*, respectively, on PDA.

[b] The data are averages ± standard errors of the means for six determinations that were acquired from three separate experiments.

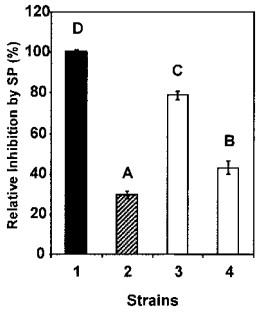

FIG. 3. Relative inhibition of *B. megaterium* due to syringopeptin (SP) secretion by *P. syringae* pv. syringae mutant strains. A bioassay for syringopeptin was performed by incubating the strains on PDA containing 0.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG) for 2 days, followed by overspraying with *B. megaterium*. The zones of inhibition of *B. megaterium* caused by strain BR132-HK4 (a *syrB1 pseC* double mutant) were compared to those caused by strain BR132 (a *syrB1* mutant) (38). Differences between treatments were determined by Tukey's W procedure ($\alpha = 0.05$) (48). Bar 1, mutant strain BR132; bar 2, BR132-HK4; bar 3, BR132-HK5 carrying pHK115 (*pseABC* efflux genes); bar 4, BR132-HK4 carrying pHK116 (pHK115 carrying a site-directed mutation in the *pseC* gene).

involved in a reversible conformational change that opens and closes a transport channel (51). Motif B and motif D were in TMS6 and TMS4, respectively, of the PseC protein, and they are proposed to be involved in proton transfer (Fig. 2) (51). Finally, motif C was in TMS11 of the PseC protein, and it has been suggested that it dictates the direction of transport (51). Two aspartic acid (D) residues and a lysine (K) residue, possible candidates for proton-translocating pathways (40), were conserved in TMS4 and TMS10, respectively, of the RND transporters, including the PseC protein (Fig. 2). Twelve TMSs and two large hydrophilic loops were predicted to be located in the PseC protein, based on hydropathy analysis (51) and multiple-alignment analysis with known RND transporters, such as the AcrB, AcrD, and AcrF proteins (16).

Screening for secretion of syringomycin and syringopeptin by the *pseABC* mutants. Strains B301D-HK2 (a *pseA* mutant), B301D-HK3 (a *pseB* mutant), and B301D-HK4 (a *pseC* mutant) were screened for secretion of syringomycin and syringopeptin on PDA using *G. candidum* and *B. megaterium* as the indicator microorganisms, respectively (58). All three mutants showed reduced zones of inhibition ($P < 0.05$) of *G. candidum* that were between approximately 40% and 45% (radius, 6 mm) less than those observed with parental strain B301D (radius, 11 mm) (Table 3); results for mutant strains B301D-HK2 and B301D-HK3 are not shown. The zones of inhibition of *B. megaterium* by mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 appeared to be between approximately 50% and 60% (radius, 4 to 5 mm) of the zones of inhibition ($P < 0.05$) observed with parental strain B301D (radius, 9 mm) (Table 3); results for mutant strains B301D-HK2 and B301D-HK3 are not shown. However, mutant strains B301D-HK2, B301D-HK3, and B301D-HK4 that carried pHK115 produced large zones, and the average radii of the zones of inhibition of *G. candidum* and *B. megaterium* were approximately 9 and 8 mm, respectively (Table 3). These data indicated that there was complementation of the *pseA*, *pseB*, and *pseC* mutations by pHK115, which carried the *pseABC* efflux genes. Based on a previous study which reported that syringomycin inhibits the growth of *B. megaterium* in PDA plate bioassays (58), strain BR132-HK4 (a *pseC* and *syrB1* double mutant) was generated to exclude the effect of syringomycin on bioassays for syringopeptin secretion. Mutant strain BR132-HK4 was screened for syringopeptin secretion by a PDA plate bioassay with *B. megaterium*, and it was observed to form zones of inhibition (radius, 4 mm) that were approximately 70% smaller than those observed for strain BR132 (*syrB1* mutant; radius, 12 mm) (Fig. 3).

The conserved D and K residues in the RND transporter family have been reported to have an essential role in the export of substrates based on functional studies with the AcrB and MexB RND transporters (24). The conserved K residue found in the PseC protein (Fig. 2) was tested to see whether it had a significant role in syringopeptin secretion. Plasmid pHK116 was generated by replacing Lys^{906} with Asp in the product of the *pseC* gene of pHK115, and then it was introduced into mutant strain BR132-HK4 to determine the functional importance of the Lys^{906} residue of the PseC protein for syringopeptin secretion (Fig. 3). Mutant strain BR132-HK4 carrying pHK116 produced zones of inhibition of *B. megaterium* with radii of approximately 5 mm, while mutant strain BR132-HK4 carrying pHK115 produced larger zones of inhibition (radii, approximately 8 mm). In comparison, strain BR132 (a *syrB1* mutant) produced zones of inhibition with an average radius of 11.5 mm. Therefore, these data showed that the Lys^{906} residue of the PseC protein was functionally important in syringopeptin secretion.

Effect of the *pseC* mutation on expression of the syringomycin and syringopeptin synthetase genes. Quantitative real-time RT-PCR was used to determine the effect of the *pseC* mutation on transcript levels of the syringomycin (*syrB1*) and syringopeptin (*sypA*) synthetase genes (7). Expression of the synthetase genes was compared for strain B301D-HK4 (a *pseC* mutant) and parental strain B301D following culture of these strains on SRM_{AF} medium for 72 h (39). The transcript levels of the *syrB1* and *sypA* genes in mutant strain B301D-HK4 were approximately 61% and 15%, respectively, of those in parental strain B301D ($P < 0.05$) (Fig. 4). The results demonstrate a substantial reduction in *sypA* expression in *pseC* mutant strain B301D-HK4.

Virulence of the *pseC* mutant for cherry fruits. The virulence of strains B301D-HK2 (a *pseA* mutant) and B301D-HK4 (a *pseC* mutant) was determined with immature Bing cherry fruits

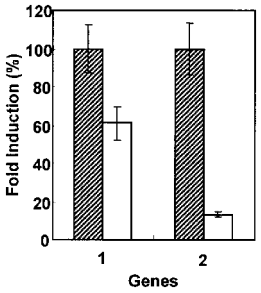

FIG. 4. Effects of the *pseC* mutation on expression of the *syrB1* (38) and *sypA* (58) synthetase genes. Expression of the synthetase genes was compared for strain B301D-HK4 (*pseC* mutant) (open bars) and parental strain B301D (cross-hatched bars) using quantitative real-time RT-PCR. Relative differences were measured (4). The relative levels of mRNA were determined from the threshold values that were normalized for 16S rRNA gene expression (endogenous control), and then the wild-type (parental strain B301D) value was defined as 100%. The error bars indicate the standard errors of the means. The relative levels of expression of the *syrB1* (bars 1) and *sypA* (bars 2) genes by mutant strain B301D-HK4 were compared with the levels of expression by parental strain B301D ($\alpha = 0.05$).

using methods described previously (52). The lesion diameters were used to quantify the relative virulence for the cherry fruits. Mutant strains B301D-HK2 and B301D-HK4 produced lesions that were nearly 2.3 mm and 2.1 mm in diameter, which were approximately 46% and 42% ($P < 0.05$), respectively, of the diameters of the lesions formed by parental strain B301D (5.0 mm). In comparison, the average virulence shown by mutant strain BR132 (3.7 mm) was approximately 74% of that observed for parental strain B301D.

Control of expression of the *pseABC* efflux genes by the GacS/GacA two-component system. A translational fusion of the *pseA* gene with the *uidA* gene encoding β-glucuronidase was used as a reporter to determine the regulatory relationship between the GacS/GacA system and *pseA* expression. Plasmid pHK92, which contained the *pseA*::*uidA* reporter construct, was introduced into parental strain B301D, strain B301D-HK5 (a *gacA* mutant), and strain B301D-SL7 (a *salA* mutant). Mutant strains B301D-HK5 and B301D-SL7 carrying pHK92 exhibited GUS activities of approximately 340 and 234 U per 10^8 CFU, respectively, which were approximately 15% of the GUS activity ($P < 0.05$) expressed by parental strain B301D (2,742 U per 10^8 CFU) (Fig. 5).

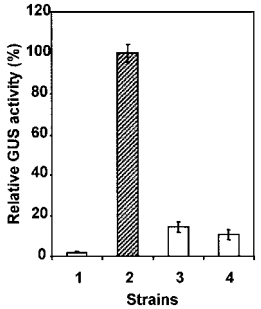

FIG. 5. Effects of the *gacA* and *salA* mutations on expression of the *pseA*::*uidA* reporter construct. The *pseA*::*uidA* reporter construct was inserted into the SmaI site of pUCP26 (47) in the reverse orientation with respect to the *lacZ* promoter of pUCP26, which generated pHK092. The strains were transformed with pHK092, incubated on PDA for 3 days, and then tested for GUS activity. The measured GUS activities of strains B301D-HK5 (a *gacA* mutant) and B301D-SL7 (a *salA* mutant) were compared to that of parental strain B301D. The error bars indicate the standard errors of the means. Bar 1, B301D with pUCP26 (vector); bar 2, B301D with pHK092; bar 3, B301D-HK5 with pHK092; bar 4, B301D-SL7 with pHK092 ($\alpha = 0.05$).

Antibiotic susceptibility of *pseC* mutant. Antimicrobial susceptibility tests were performed to determine whether the PseABC efflux system contributed to antibiotic resistance in *P. syringae* pv. syringae strains. Mutant strain B301D-HK4 and parental strain B301D exhibited the same susceptibilities to all the antibiotics tested; the MICs of acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, gentamicin, and tetracycline were 12.5 μg/ml, 100 μg/ml, 200 μg/ml, 50 μg/ml, 12.5 μg/ml, 6.3 μg/ml and 0.2 μg/ml, respectively. In comparison, the MICs for *P. aeruginosa* PAO1 were similar to those reported in previous studies (2, 36, 42); the MICs of acriflavine, aztreonam, carbenicillin, chloramphenicol, erythromycin, and gentamicin were 200 μg/ml, 50 μg/ml, 50 μg/ml, 200 μg/ml, 100 μg/ml, 12.5 μg/ml, and 3.1 μg/ml, respectively.

In order to define the functional relationship of the PseABC efflux system with the *E. coli* AcrAB-TolC efflux system (46), *E. coli* strain AG100A (an *acrB* mutant) was transformed with pJS115 and then assessed for susceptibility to acriflavine, erythromycin, and tetracycline. Heterologous expression of the *pseABC* efflux genes increased the resistance of mutant strain AG100A to the antibiotics tested. However, the heterologous expression was not enough to restore full resistance of mutant strain AG100A. The resistance to acriflavine, erythromycin, and tetracycline was two- to fourfold greater in mutant strain AG100A expressing the *pseABC* efflux genes carried in a pBSK vector than in mutant strain AG100A carrying only a pBSK vector. The MICs of acriflavine, erythromycin, and tetracycline for mutant strain AG100A carrying pJS115 were 3.12 μg/ml, 50 μg/ml, and 0.78 μg/ml, respectively, whereas the MICs of acriflavine, erythromycin, and tetracycline for mutant strain AG100A carrying only a pBSK vector were 1.56 μg/ml, 25 μg/ml, and 0.19 μg/ml respectively. In comparison, the MICs of acriflavine, erythromycin, and tetracycline for mutant strain AG100A without either a pBSK vector or pJS115 were 3.12 μg/ml, 25 μg/ml, and 0.39 μg/ml, respectively. Furthermore, the MICs of acriflavine, erythromycin, and tetracycline for parental strain AG100 were 25 μg/ml, 200 μg/ml, and 1.56 μg/ml, respectively.

DISCUSSION

P. syringae pv. syringae strain B301D secretes two major phytotoxins, syringomycin and syringopeptin (7). Previous studies (21, 52) suggested that phytotoxin secretion is greatly facilitated by the SyrD efflux system, although a *syrD* mutation failed to cause a complete loss of the ability to secrete syringomycin and syringopeptin. During further sequencing of the *syp* gene cluster, another transporter system was identified at the left border of the *syp* gene cluster (60), and this system was called the PseABC efflux system. The predicted PseA protein was homologous to a probable RND outer membrane protein (*R. solanacearum*) (55), the OprM protein (*P. aeruginosa*) (63), and the TolC protein (*E. coli*) (8). Furthermore, the PseA protein was observed to contain two TMSs and motifs charac-

teristic of the outer membrane protein family. The amino acid sequence of the predicted PseB protein was homologous to those of a probable RND membrane fusion protein (MPF) of *R. solanacearum* (55), the MexA protein of *P. aeruginosa* (63), and the AcrA protein of *E. coli* (8). Moreover, the PseB protein was observed to have one TMS and contained a HlyD family secretion protein motif that is characteristic of the membrane fusion protein family. The predicted PseC protein was homologous to a putative RND transporter from *R. solanacearum* (55), the MexB protein of *P. aeruginosa* (63), and the AcrB protein of *E. coli* (8). In addition, the PseC protein was observed to contain 12 TMSs, two large periplasmic loops, and four motifs (motifs A, B, C, and D) characteristic of the RND-type transporter family. When substrates are transported, an outer membrane protein, a membrane fusion protein, and an RND transporter form a functional three-component complex, such as the AcrAB-TolC and MexAB-OprM efflux systems (71). Similarly, the PseA, PseB, and PseC proteins are expected to form a functional three-component complex that secretes the lipopeptide phytotoxins. In summary, the PseABC efflux system was classified as a member of the RND transporter family that has a critical role in the lipopeptide phytotoxin secretion and virulence of *P. syringae* pv. syringae B301D.

The RND superfamily is composed of eight recognized phylogenetic families, which are correlated with substrate specificity (53). In gram-negative bacteria, RND families 1 to 3 form a three-component efflux system conjugated with a membrane fusion protein and an outer membrane protein. Family 1 is involved in the export of heavy metals (15), family 2 is involved in the export of multiple drugs (41), and family 3 is involved in the export of lipooligosaccharides involved in plant nodulation by rhizobia (49). Based on these criteria, the PseABC efflux system appeared to be a unique RND-type transporter system in that it secretes specific lipopeptide phytotoxins, which are natural secondary metabolites produced by a gram-negative bacterium, *P. syringa*e pv. syringae (22). However, to support the uniqueness of the PseABC efflux system, it is necessary to determine whether other substrates are secreted by the PseABC efflux system.

Strain B301D-HK4 (a *pseC* mutant) was as sensitive to a series of antibiotics as parental strain B301D in MIC tests. These results indicate that the PseABC efflux system was not involved in altering the resistance of strain B301D of *P. syringae* pv. syringae to the antibiotics tested. Thus, we propose that PseC might have several homologs in the B301D genome or another efflux system might be responsible for conferring resistance to antibiotics. Because of the absence of a complete genome sequence for strain B301D, the draft sequence of the *P. syringae* pv. syringae strain B728a genome was searched for a PseC homolog (http://genome.jgi-psf.org/draft_microbes/psesy/psesy.home.html). Only one PseC homolog (96.4% identity; Z score, 207) was found in the B728a genome. Nonetheless, it is not known how many genes encode RND-type transporters in the B728a genome. Thus, the annotated genomes of *P. syringae* pv. tomato strain DC3000 (10), *Pseudomonas putida* strain KT2440 (43), and *P. aeruginosa* strain PAO1 (63) were searched for genes encoding members of the RND-type transporter family. Interestingly, multiple genes encoding RND-type transporters were found in these genomes; nine genes encoding probable RND-type transporters were found in the DC3000 genome, 19 genes were found in the KT2440 genome, and 17 genes were found in the PAO1 genome (53). Furthermore, most of the RND-type transporters were predicted to be multidrug exporters (53). Therefore, it was speculated that, in addition to the *pseC* gene, genes encoding additional RND-type transporters likely exist and their products might be responsible for the export of antibiotics and metabolites in strain B301D.

Heterologous expression of the *pseABC* efflux genes in *E. coli* indicated that the PseABC efflux system might have patterns of substrate specificity similar to those of the AcrAB-TolC efflux system (46). Although the expression of *pseABC* efflux genes in *E. coli* strain AG100A (an *acrB* mutant) failed to fully restore resistance to acriflavine, erythromycin, and tetracycline due to the *acrB* mutation, expression of the *pseABC* efflux genes partially increased the antibiotic resistance in mutant strain AG100A. These results indicated that the PseABC efflux system has partial substrate specificity for acriflavine, erythromycin, and tetracycline, which were major substrates for the AcrAB-TolC efflux systems (46). Furthermore, to confirm that the PseC protein has a secondary structural relationship to RND-type transporters, site-directed mutagenesis was performed to replace Lys^{906} with Asp in the PseC protein. Plasmid pHK116 that produced a PseC protein harboring the the Lys^{906} replaced by Asp failed to fully complement the ability to secrete syringopeptin. Thus, replacement of the Lys residue in PseC protein disturbs the inhibition to *B. megaterium* by syringopeptin. Similar results were observed for replacement of the corresponding Lys residues in AcrB (16) and MexB (24). This demonstrates that there might be similarities in substrate specificity and secondary structure between the PseC protein and RND transporters, as exemplified by AcrB.

Expression of the *pseA*::*uidA* reporter was reduced 85% in strains B301D-HK5 (a *gacA* mutant) and B301D-SL7 (a *salA* mutant) compared to parental strain B301D, which indicated that expression of the *pseABC* efflux genes was controlled by a GacS/GacA two-component system. The sensor kinase GacS and the response regulator GacA trigger signal transduction to express genes required for the biosynthesis of secondary metabolites, including phytotoxins (25). *P. syringae* pv. syringae strain B728a uses the GacS/GacA two-component system to control production of syringomycin, extracellular polysaccharide, and proteases that are involved in virulence (30). The SalA transcriptional regulator is essential for syringomycin production and lesion formation by strains B728a and B301D (30, 35). A recent study demonstrated that expression of all ORFs in the *syr-syp* genomic island is controlled by SalA (35a). Thus, the GacS/GacA two-component system is known to control syringomycin production and lesion formation by regulating expression of the *salA* gene (30). However, it is not verified whether the GacS/GacA two-component system or the SalA protein controls expression of the *pseABC* efflux genes required for secretion of syringomycin and syringopeptin. Results of this study demonstrated that expression of the *pseA*::*uidA* reporter construct was significantly reduced in both *salA* and *gacA* mutants. These data showed that the GacS/GacA two-component system controls expression of the *pseA* gene through *salA* expression. This indicated that there is coregulation of the phytotoxin synthetase genes and the *pseABC*

efflux genes by the GacS/GacA two-component system. This coregulation is likely to help cells balance phytotoxin biosynthesis and secretion.

As shown previously, expression of the *syrB1* gene is reduced in strain BR105 (a *syrD* mutant) (52), and a mutation in the *pseC* gene caused a significant reduction in expression of the *syrB1* and *sypA* genes. Expression of the *sypA* gene in mutant strain B301D-HK4 was greatly decreased compared to that in parental strain B301D (85% reduction). In addition, there was a significant decrease in expression of the *syrB1* gene in mutant strain B301D-HK4 (39% reduction). These results demonstrated that mutations in the *pseC* or *syrD* gene reduce the transcript levels of the syringomycin and syringopeptin synthetase genes, which subsequently results in reduced secretion of the phytotoxins. Previous studies demonstrated that end products can regulate secondary metabolite production (17, 66). For example, the HlyBD-TolC efflux system secretes hemolysin A across both membranes of *E. coli* (17). The efflux system consists of a cytoplasmic membrane protein of the ABC transporter system (HlyB), a membrane fusion protein (HlyD), and an outer membrane protein (TolC) (17). It has been observed that the total intracellular levels of hemolysin are low in *hlyB*, *hlyD*, and *tolC* mutants (67). This indicates that there is regulatory coupling between hemolysin production and toxin secretion. Correspondingly, a regulatory coupling between syringomycin and syringopeptin production and secretion may exist. A mutation in the *pseC* gene interfered with secretion of syringomycin and syringopeptin and caused reduced expression of the *syrB1* and *sypA* genes.

The crystal structure of AcrB revealed the presence of a TolC docking domain in the extramembrane headpiece of the AcrB protein, which signifies a direct interaction between the TolC and AcrB proteins (40). Thus, the AcrAB-TolC efflux system transports substrates through a membrane-spanning conduit formed by this interaction. Correspondingly, the PseC protein (RND-type transporter) might contain a docking domain for the PseA protein (outer membrane protein). A direct interaction between the PseC and PseA proteins is likely to occur and form a membrane-spanning transit pathway. Thus, the PseABC efflux system is expected to secrete lipopeptide phytotoxins through the transit pathway with preference for syringopeptin in *P. syringae* pv. syringae.

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